

# **Expression of CD163 on Bovine Alveolar Macrophages and Peripheral Blood Mononuclear Cells**

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## **Abstract**

The Agricultural Research Center of the United States Department of Agriculture identifies bovine respiratory disease (BRD) as the most common and costly disease of feedlot cattle in the United States (1). The alveolar macrophage serves as the first line of defense for the lung alveolus by recognition, phagocytosis, and destruction of the bacterial agents reaching the alveolus.

CD163 is a monocyte/macrophage-restricted, cysteine-rich, scavenger receptor that binds and internalizes circulating haptoglobin-hemoglobin (Hp-Hb) complexes (5). Macrophages expressing high levels of CD163 appear to be present in the resolution phase of inflammation (7).

The purpose of this study was to further our understanding of the cellular regulation of host inflammatory mediators in bovine respiratory diseases by determining the expression of CD163 on alveolar macrophages and peripheral blood mononuclear derived macrophages.

Bovine peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of healthy calves and cultured for differentiation into macrophages. After cellular differentiation, the cultured cells and freshly isolated alveolar macrophage cells obtained by bronchoalveolar lavage were labeled with anti-CD163 antibodies and evaluated for expression of CD163 by flow cytometry. Positive expression of CD163 (61.83%) was observed on alveolar macrophages. These findings demonstrate that bovine macrophages do express a surface protein similar to CD163. Nevertheless, neither the peripheral blood monocytes nor the monocyte-derived macrophages expressed CD163 in any of our trials. Four of the six flow cytometry runs also did not reveal a high level of monocyte-

macrophage differentiation, however, with the modification of our isolation and culturing techniques, the final two flow cytometry runs demonstrated higher levels of cellular differentiation.

Additional studies are needed to determine expression of CD163 on macrophages derived from peripheral blood mononuclear cells, and alternative methods of differentiation will be necessary to reproducibly induce CD163 expression and further the understanding of the CD163-associated, anti-inflammatory pathway.

## **Introduction**

The Agricultural Research Center of the United States Department of Agriculture identifies bovine respiratory disease (BRD) as the most common and costly disease of feedlot cattle in the United States (1). More beef cattle develop and subsequently die from respiratory diseases than from any other cause, costing over one billion dollars annually to the cattle industry (2). We now know that bovine respiratory diseases are caused by the presence of one or more pathogenic organisms, and the manifestation of this clinical disease is caused by a complex interaction of various host, pathogen, and environmental factors (3).

Monocytes and macrophages are prominent cells at sites of chronic inflammation (4). The alveolar macrophage specifically serves as the first line of defense against bovine respiratory disease by the recognition, phagocytosis, and destruction of bacterial agents reaching the alveolus. In the presence of more virulent organisms, the alveolar macrophage recruits other inflammatory cells to the lung in order to limit microbial infection.

CD163 is a monocyte/macrophage-restricted, cysteine rich protein, scavenger receptor that binds and internalizes circulating haptoglobin-hemoglobin (Hp-Hb) complexes (5). CD163 action on the haptoglobin-hemoglobin complex fuels an anti-inflammatory response mediated by heme metabolites. Monocytes express CD163 constitutively at low levels, but expression increases during macrophage differentiation (6). Macrophages expressing high levels of CD163 appear to be present in the resolution phase of inflammation (7), and are therefore thought to play an important role in bovine respiratory disease. CD163 is an attractive candidate for potential diagnostic use as a marker of monocyte/macrophage activity because of its apparent roles in the anti-inflammatory response. Nevertheless, it is unclear how many or what kinds of circulating monocytes express the CD163 molecule on their surface (5).

## **Objective**

Identify and determine expression of CD163 in bovine alveolar macrophages and macrophages derived from bovine peripheral blood mononuclear cells in order to further the understanding of the cellular regulation of host inflammatory mediators in bovine respiratory diseases and possibly assist in the treatment and prevention of bovine respiratory diseases.

## **Materials and Methods**

### **Isolation and Incubation of Monocytes for Tissue Cell Culture**

Approximately 50 milliliters of whole blood were drawn from healthy calves available for research at The Ohio State University Veterinary Teaching Hospital. The

plasma was removed from the blood and the buffy coat (a layer that includes monocytes and leukocytes) was diluted with phosphate buffer solution or Hank's buffered salt solution. Histopaque was used to separate cells and isolate the monocytes for culture. After washing the cells with phosphate buffered solution, 10 microliters of cells were diluted with 90 microliters of phosphate buffered solution and counted on a hemocytometer. Cells were also stained and examined under a microscope for type and quality. After being counted on the hemocytometer, monocytes were diluted to  $1 \times 10^6$  cells/mL and placed into culture wells with a cell media culture made up of RPMI-1640 with 2mM L-glutamine, 1.5% sodium bicarbonate, 4.5 g./L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% fetal bovine serum to promote differentiation into macrophages. Cells were cultured at 37° Celsius in a 5% CO<sub>2</sub> incubator for approximately eleven days. Twenty-four hours after being initially placed into culture, 75% of the cell media was removed from the culture and cells were washed with phosphate buffered solution before being placed back into culture with new media. This process was repeated every three to five days until day eleven. Cell culture plates were also examined under a microscope to monitor any changes in cell size, number, or condition. Some cell cultures were treated with a 1/1000 dexamethasone/RPMI cell culture media solution to encourage up-regulation of CD163 expression.

#### Removal and Labeling of Monocyte-Derived Macrophages with Anti-CD163 Antibodies

On day eleven, the cells were removed from the culture by pipetting out the old media and adding cold phosphate buffered solution to the cell culture wells for ten minutes before scraping the cell culture wells with cell scrapers. The remaining cells and media were then recounted on the hemocytometer and re-diluted to  $1 \times 10^6$  cells/mL. One

milliliter of this solution was added to each flow cytometry tube and centrifuged to form a small pellet of cells. The liquid was removed and 100 microliters of phosphate buffered solution with 20mM glucose and 1% bovine serum albumin was added to each tube and the appropriate volume of Serotec antibodies were added to each tube. Primary antibodies used were Serotec Mouse Anti-Porcine. The isotype/negative control antibody was a Serotec IgG1 Negative Control, and the secondary antibody used was a Goat Anti-Mouse IgG (H & L) (Multi-species absorbed): FITC antibody. The appropriate antibodies were added to each tube (2 primary, 1 isotype/negative control, 1 no primary antibody, and 1 untreated tube) and incubated at room temperature for 30 minutes. After incubation cells were again washed with phosphate buffered solution with glucose and bovine serum albumin. Secondary antibody was then added to the appropriate tubes and cells were cultured again, this time in the dark, at room temperature for 30 minutes. Following a final washing in the phosphate buffered solution, cells were resuspended to 500 microliters with either phosphate buffered solution with glucose and bovine serum albumin or 0.5% paraformaldehyde in phosphate buffered solution. Cells placed in the paraformaldehyde solution could incubate for up to one week in the 4° Celsius refrigerator. Final cell solutions were taken to the ImCore Cytometry Lab at the Center for Retrovirus Research in Goss Laboratories of The Ohio State University. Flow cytometry was run by a technician, Elizabeth Wheeler, and the resulting data was shared.

Alveolar macrophage cells were also harvested on two occasions from healthy, anesthetized calves by bronchoalveolar lavage conducted by Dr. Jeff Lakritz, DVM of The Ohio State University Veterinary Teaching Hospital. These cells were also washed in

phosphate buffered solution and labeled with the anti-CD163 antibodies in the same way the cultured monocyte-derived macrophages were labeled.

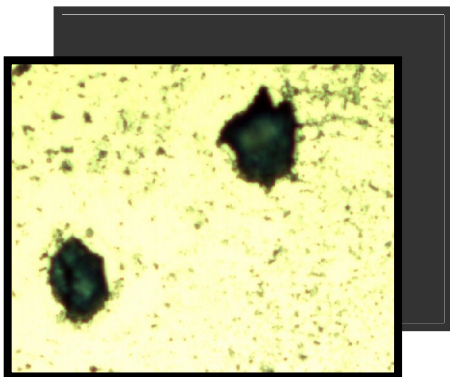
## **Results**

Flow cytometry was run on six groups of cultured, differentiated peripheral blood mononuclear cells and bovine alveolar macrophages for a total of forty-three samples. Cell samples in four of the six flow cytometry runs did not reveal a high enough level of monocyte-macrophage differentiation to identify an absence of the CD163 response. After researching alternative monocyte culturing techniques, we were able to obtain a higher percentage of differentiated cells.

Positive expression of CD163 (61.18%) was found on the alveolar macrophage cells isolated by bronchoalveolar lavage. These findings demonstrate that bovine macrophages from the lungs do express a surface protein that is at least similar to CD163. These findings also confirmed the potential of the anti-CD163 anti-porcine antibodies we had chosen.

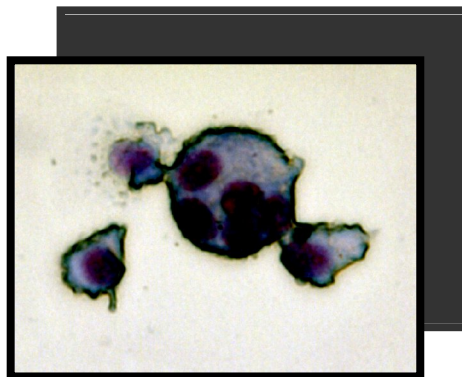
Nevertheless, neither the undifferentiated peripheral blood monocytes nor the monocyte-derived macrophages expressed significant levels of CD163 fluorescence in any of our trials.





**Figure 1**

**Figure 1** – Undifferentiated bovine peripheral blood mononuclear cells.



**Figure 2**

**Figure 2** – Bovine peripheral blood mononuclear cells that have differentiated into macrophages.

## **Flow Cytometry Data Results**

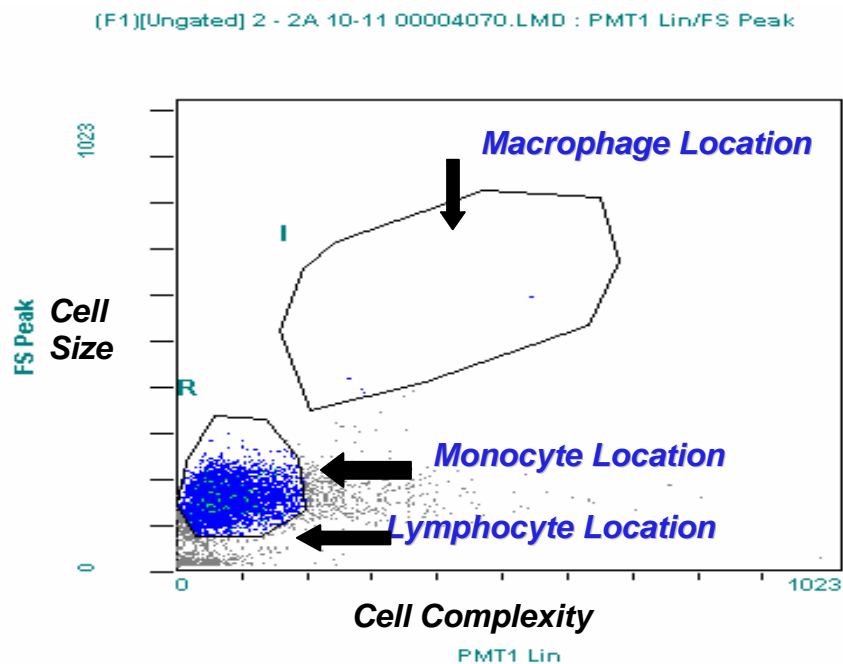
The following data was selected from three of our most representative flow cytometry sample results. The data demonstrates, respectively, very little monocyte differentiation and no CD163 expression, monocyte differentiation into macrophages with insignificant levels of CD163 expression, and alveolar macrophages demonstrating a significant level of cellular differentiation and CD163 expression.

The scatter graph axes and expected cellular location and differentiation are defined in Figure #3 and can also be applied to Figure #5 and Figure #7. Figures #4, #6, and #8 measure the cellular level of fluorescence on a log scale. The blue peaks demonstrate the location(s) of the highest levels of fluorescence. The desired data on these graphs would include two peaks, with the largest shifted toward the middle or right of the graph area. Insignificant levels are seen in Figures #4 and #6, and Figure #8 demonstrates more desirable flow cytometry data.

**Figure #3**

**Scatter Plot**

*Cultured peripheral blood mononuclear cells demonstrating very little differentiation from monocytes into macrophages and no CD163 expression.*

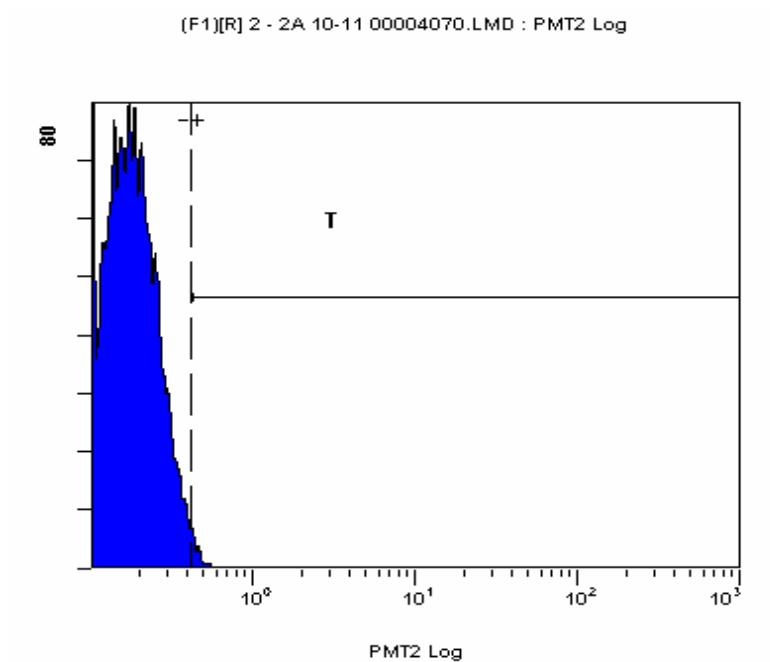


Region	Number	%Total	%Gated	X-Mean
I	9	0.07	0.07	342.1
R	10,000	82.78	82.78	72.6

**Figure #4**

**Fluorescence Log Scale**

***Cultured peripheral blood mononuclear cells demonstrating negligible CD163  
expression by fluorescence.***

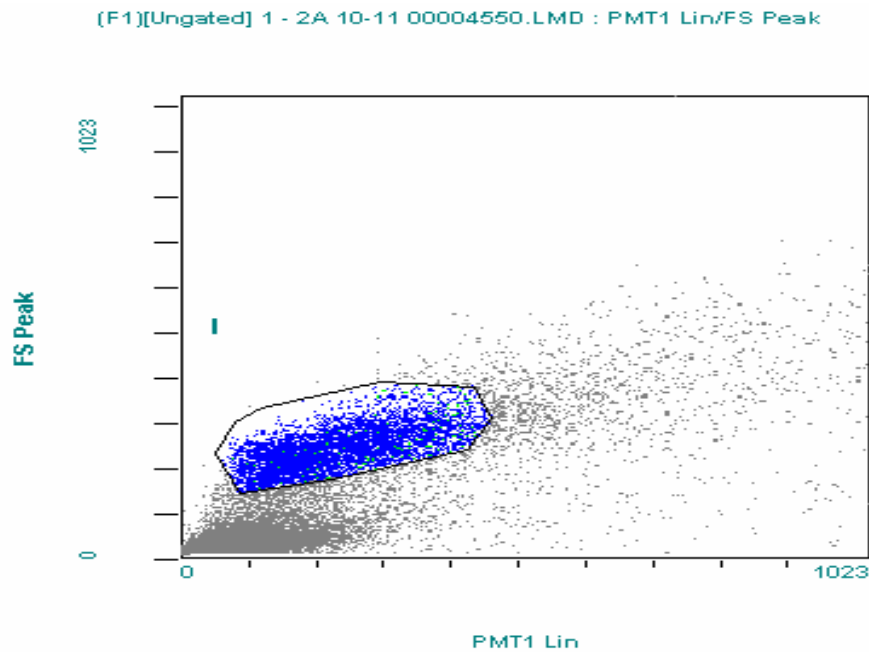


Region	Number	%Total	%Gated	X-Mean
T	120	0.99	1.20	0.7

**Figure #5**

**Scatter Plot**

*Cultured peripheral blood mononuclear cells demonstrating a high level of monocyte differentiation into macrophages, but insignificant CD163 expression.*

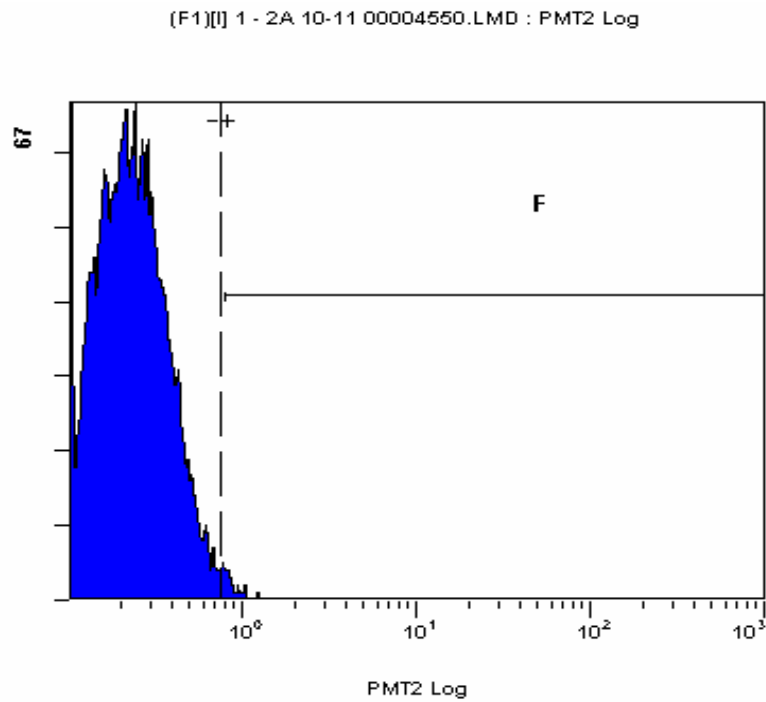


Region	Number	%Total	%Gated	X-Mean
I	10,000	9.47	9.47	236.0

**Figure #6**

**Fluorescence Log Scale**

***Cultured peripheral blood mononuclear cells demonstrating negligible CD163 expression.***

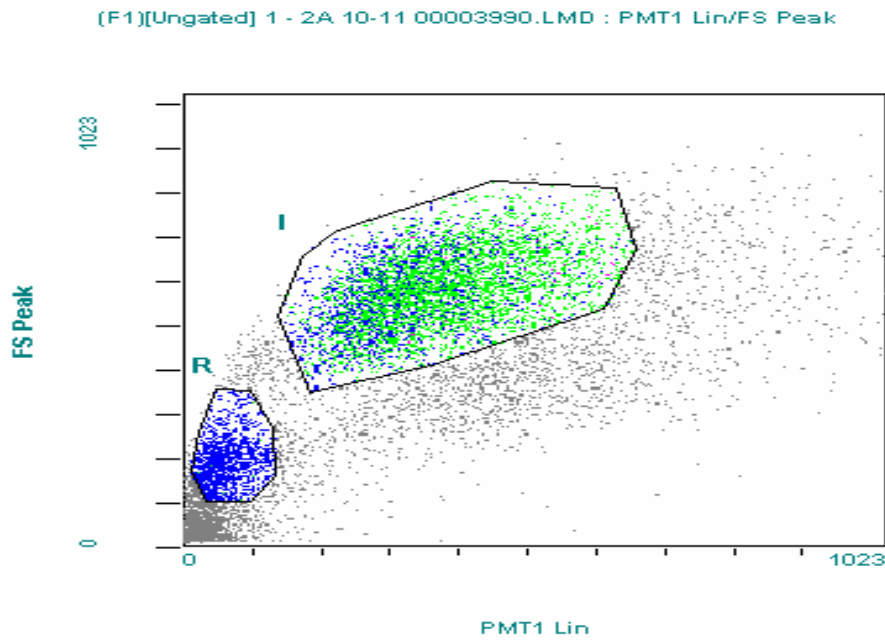


Region	Number	%Total	%Gated	X-Mean
F	217	0.21	2.17	1.7

**Figure #7**

**Scatter Plot**

*Alveolar macrophage cells obtained by bronchoalveolar lavage demonstrating a high level of cellular differentiation and positive CD163 expression by fluorescence.*

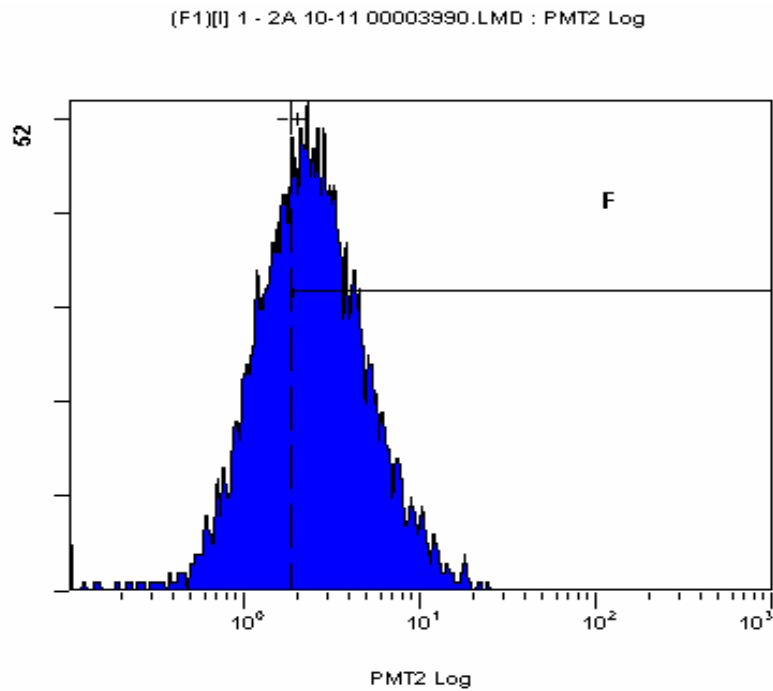


Region	Number	%Total	%Gated	X-Mean
I	10,000	46.94	46.94	364.7
R	2571	12.07	12.07	61.0

**Figure #8**

**Fluorescence Log Scale**

*Cultured peripheral blood mononuclear cells demonstrating positive CD163 expression  
by fluorescence (61.18% and shifted peak).*



Region	Number	%Total	%Gated	X-Mean
F	6118	28.72	61.18	5.0

## **Implications and Conclusion**

Previous studies have shown that Interleukin-10 (IL-10) and glucocorticoids may induce higher CD163 expression on cultured monocytes. The fact that CD163 can be up-regulated by potent anti-inflammatory mediators such as glucocorticoids and IL-10 is a strong indicator that CD163 may be an important anti-inflammatory molecule and a potential biomarker for inflammation and inflammatory conditions (8). Based on the inconclusive results of our study, however, additional studies will be needed to determine expression of CD163 on macrophages derived from peripheral blood mononuclear cells.

Alternative methods of differentiation are suggested in order to reproducibly induce CD163 expression and further the understanding of the CD163-associated, anti-inflammatory pathway. Some possible modifications that may assist in determining the expression of CD163 include treating cell cultures with Interleukins (IL-6 or IL-10) to encourage up-regulation of the anti-inflammatory response or possibly culturing peripheral blood mononuclear cells with the CD163 positive alveolar macrophage cells to induce CD163 expression. Another suggested possibility is to use haptoglobin bound to hemoglobin in an attempt to induce binding and stimulation of the possible CD163 on macrophages.

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